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New reports of *Gymnopus* from Pakistan based on ITS sequences

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Abstract — Specimens of *Gymnopus luxurians* and *G. menehune* from Pakistan are characterized morphologically and using nrDNA internal transcribed spacer sequences. These fungal species represent new records for South Asia. A key to the *Gymnopus* species known from Pakistan is provided.

Key words — Abbottabad, *Collybia*, Khyber Pakhtoonkhaw, *Marasmiaceae*, saprobic fungi

Introduction

Gymnopus (Pers.) Roussel was monographed by Antonín & Noordeloos (1997) and many mushrooms from *Collybia* sections *Vestipedes* and *Levipedes* have now been transferred into this genus (Antonín & Noordeloos 1997). *Gymnopus* consists of delicate to semi-fleshy mushrooms commonly found on leaf and woody litter in tropical to temperate ecosystems, and *Gymnopus* sect. *Vestipedes* is characterised by a hairy or tomentose stipe surface and a simple pileus epicutis without a Rameales- or Dryophila-structure (Antonín & Noordeloos 1997). Ecologically, *Gymnopus* species are considered important in recycling nutrients (Singer 1986; Mata & Ovrebo 2009).

Five *Gymnopus* species have previously been reported from Pakistan (under their *Collybia* synonyms): *Gymnopus confluens* (Pers.) Antonín et al., *G. dryophilus* (Bull.) Murrill, *G. fusipes* (Bull.) Gray, *G. erythropus* (Pers.) Antonín et al. (as *C. kuehneriana*), and *G. peronatus* (Bolt.) Gray (Ahmad 1980; Iqbal & Khalid 1996; Shibata 1992; Sultana et al. 2011). During a collecting trip for macrofungi in Khyber Pakhtoonkhaw in 2012, many mushroom species were collected. Amongst them, three specimens belonging to *Gymnopus* sect. *Vestipedes* were identified as *G. luxurians* and *G. menehune*, which are new records from subtropical pine forests of Pakistan.

Table 1. ITS-rDNA sequences of *Gymnopus* and *Omphalotus* spp. used in the phylogenetic analysis

Taxon	Country	Genbank #	Collection/ Voucher #
G. biformis	Costa Rica	DQ450064	TFB7843
	Costa Rica	DQ450063	TFB7820
G. confluens	Russia	AY256697	TENN58242
	—	HM240527	UBC F19677
G. cylindricus	Costa Rica	AY256696	TENN58024
	Costa Rica	DQ450057	TENN58024
G. dryophilus	Italy	JX536157	BRNM707149
	Czech Republic	JX536158	BRNM712600
G. erythropus	Slovakia	DQ449996	SAV XI2002
	USA	DQ449998	WTU JFA12910
G. fibrosipes	Costa Rica	AF505763	TENN56660
	—	AY842953	PR23TN
G. fusipes	France	AY256710	TENN59217
	Austria	AF505777	TENN59300
G. gibbosus	—	AY263437	AWW66
	—	AY263438	AWW95
G. luxurians	Pakistan	KF803760	MSM#001
	Pakistan	KF803761	MSM#002
	USA	AY256709	TENN57910
	Switzerland	DQ450022	TENN50619
G. menehune	Pakistan	KF803762	MSM#003
	—	JN182864	Not given
	—	AY263426	DED5866
G. mesoamericanus	Costa Rica	DQ450036	TENN58106
	Costa Rica	AF505768	NYBG REH7379
G. polygrammus	Puerto Rico	DQ450028	TENN56589
	Not given	AY842954	PR 2542TN
G. readiae	New Zealand	DQ450034	TENN53687
	New Zealand	HQ533036	PDD95844
G. subcyathiformis	Puerto Rico	DQ450041	TENN58130
	Dominican Republic	DQ450042	TENN59550
G. subpruinosus	USA	DQ450026	TENN56242
	USA	DQ450027	TENN59477
O. illudens	USA	AY313271	TENN54507
O. olearius	France	AY313277	culture 9061b
O. olivascens	USA	AY313281	TENN55337

Materials & methods

Basidiomata were collected, photographed, vouchered, dried under fan heater, and characterized morphologically. Specimen sections were mounted in 5% KOH for observation under a biological microscope (MX4300H, Meiji Techno Co., Ltd., Japan). Phloxine was used to increase contrast of structures, and Melzer's reagent was used to test for dextrinoidity of basidiospores.

Measurements of anatomical features (basidiospores, basidia, cystidia, pileus hyphae, and stipe hyphae) are presented from at least 20 measurements made with an ocular micrometer and 100× oil-immersion objective; basidiospore abbreviations include x = arithmetic mean of length and width for all spores measured and Q = length divided by width. Line drawings were made with a camera lucida. Color designations are from Munsell (1975) colour system.

Genomic DNA was extracted from a small piece of pileus by a modified CTAB method (Bruns 1995). The internal transcribed spacers (ITS1 and ITS2) and 5.8S region of the nuclear ribosomal RNA gene were amplified with the primer pairs ITS1F/ITS4 (White et al. 1990; Gardes & Bruns 1993) using the Extract-N-Amp plant DNA extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). PCR was carried out under the following cycling parameters: initial denaturation (94 °C for 1 min), 35 cycles (94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min), and final extension 72 °C (8 min). Amplified PCR products were sent for purification and bidirectional sequencing to Macrogen (Korea). Two sequences each of *Gymnopus luxurians* and *G. menehune* and other related sequences were retrieved from the Genbank and aligned by Muscle using the default setting in Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al. 2011). Sequences were manually edited and assembled using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). Following Dentinger et al. (2011) for complete ITS sequences, all sequences were trimmed with the conserved motifs 5′-(…GAT) CATTA– and –GACCT (CAAA…)-3′ and the alignment portion between them was included in the analysis. All positions containing gaps and missing data were eliminated. A phylogenetic tree was constructed with the Maximum Likelihood (ML) algorithm and Jukes & Cantor (1969) model of nrITS sequences and nearest-neighbor-interchange (NNI) as ML heuristic search method using MEGA5 software (Tamura et al. 2011). Phylogeny was tested by bootstrap value of 1000 replicates, and corresponding bootstrap values >50 % are cited in the tree. Sequences of *Omphalotus* spp. were used as outgroup based on results reported by Moncalvo et al. (2002) and Mata et al. (2004).

Sequences generated for this study were submitted to Genbank. A complete list of taxa used in this phylogenetic is shown in Table 1. Percent identities (PID) and DNA divergence were calculated by DNAStar.

Taxonomy

Gymnopus luxurians (Peck) Murrill, N. Amer. Fl. 9(5): 362 (1916). Fig. 1

Pileus 19–29 mm diam, convex when young, expanding with age to plane, flat, thin (collybioid), slightly umbonate; margin recurved or uplifted, flexuous, striate; surface dull, fibrillose; brown, dark brown or reddish brown (10R5/10), margin fading to pale brown to brown or buff (10R6/12). Lamellae adnexed,

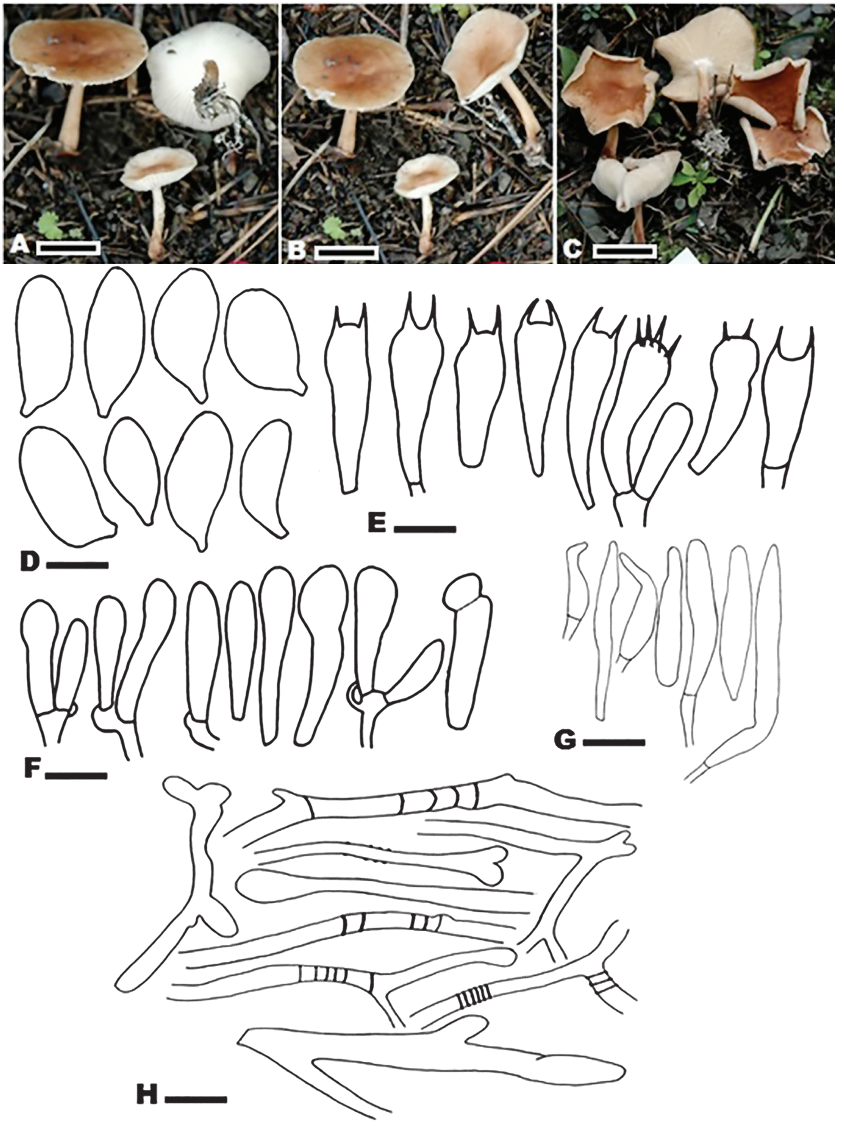

Fig. 1. *Gymnopus luxurians* (MSM#001; MSM #002). (A–C) Basidiomes. (D) Basidiospores. (E) Basidia and basidioles. (F) Cheilocystidia. (G) Caulocystidia. (H) Pileipellis. Scale bars: A–C = 2 cm; D–F = 10 µm; G–H = 20 µm.

indented or notched, close to subdistant, white at first (5YR9/2), pale grayish (5YR8/6) at maturity. Stipe 13–25 mm, central, subequal, often with subbulbous

base, hollow, striate, buff (2.5YR6/8) when young, base dark brown (10R2/6). Rhizomorphs white, numerous, branched. Odor and taste not recorded. Basidiospores 4.2–5 × 5.3–9.4 µm [x = 4.6 × 7.6 µm, Q = 1.65], oblong to ellipsoid in profile, apiculus present, smooth, thin-walled, hyaline in KOH, inamyloid. Basidia 5.6–8 × 19–28.3 µm, clavate, 2-spored basidia abundant, 4-spored also present, thin-walled, hyaline in KOH; sterigmata 2–4 µm. Basidioles subclavate, abundant. Pleurocystidia absent. Cheilocystidia 4–8 × 18–32 µm, clavate or cylindrical, hyaline, thin-walled. Pileipellis a cutis, hyphae cylindrical, 6–14 µm, thin-walled, weakly to heavily encrusted with annular brown pigments, brown in KOH. Stipe hyphae cylindrical, 5–10 µm, non-encrusted, hyaline to pale yellow in KOH. Caulocystidia cylindrical, hyaline, thin-walled 9–14 × 53–124 µm. Clamp connections present in all tissues.

Material examined: **PAKISTAN**, **Khyber Pakhtoonkhaw**, Abbottabad, Shimla, under *Pinus roxburghii* Sarg., 14 September 2012, Malka Saba & Abdul Nasir Khalid, MSM #001 (LAH; GenBank KF803760); MSM #002 (LAH; GenBank KF803761).

Comments: *Gymnopus luxurians* is a widely distributed species. It is characterized by brown or reddish brown convex pileus, slightly umbonate with striate margin, oblong to ellipsoid basidiospores, and cylindrical caulocystidia. It is frequently collected on woody debris across the continental United States and has been reported from Europe (Breitenbach & Kränzlin 1991), Hawaii (Desjardin et al. 1999), Dominican Republic (Mata et al. 2006), Costa Rica, and Panama (Mata & Ovrebo 2009). *Gymnopus luxurians* represents a new record for Pakistan.

Gymnopus luxurians falls in *G.* subsect. *Vestipedes* (Antonín & Noordeloos 1997). Other *Gymnopus* species from this subsection reported from Pakistan include *G. confluens*. (Ahmad 1980; Shibata 1992; Sultana et al. 2011), *G. peronatus* (Sultana et al. 2011), and *G. menehune* (see below). *Gymnopus confluens* is similar to *G. luxurians* in being reported from pine forests during the same season of the year but can be separated by the presence of thin and crowded lamellae and a whitish fuzz on the stipe that becomes noticeable as the fungus matures (Kuo 2013). *Gymnopus peronatus* can be distinguished by its remarkably distant gills and densely woolly-strigose stipe with white to yellow hairs (Antonín & Noordeloos 1997).

Gymnopus menehune Desjardin, Halling & Hemmes, Mycologia 91(1): 173 (1999). Fig. 2

Pileus 10–14 mm diam., convex to plano-convex and slightly umbilicate when young, infundibuliform, umbilicate at maturity, reddish brown (10R3/8) or brown fading near margin to grey or pale brown (5YR9/2); margin decurved, straight or flaring, uplifted, eroded, striate; surface dull, glabrous. Lamellae adnate to decurrent, ascending, close, with 3–6 series of lamellulae,

off-white to pale orange white (10R8/4). Stipe 23–32 mm, central, equal, hollow, pubescent to tomentose, buff color near proximal end, brown to dark brown (10R2/6) at distal end. Rhizomorphs numerous, white, branched. Odor and taste not recorded. Basidiospores elongate to ellipsoid in profile, 3–4.2(–5.4) × (–5.5)7–9 µm [x = 4.6 × 8.1 µm, Q = 1.76], smooth, thin-walled, hyaline in KOH with cytoplasmic contents, inamyloid. Basidia clavate, 6.3–7(–8) × 18.8–24 µm, commonly 2-spored, occasionally 4-spored; Sterigmata up to 4.9 µm. Basidioles clavate or fusoid, abundant. Pleurocystidia absent. Cheilocystidia broadly clavate or irregular in outline, sometimes lobed, thin-walled, 5–7 × 20–30 µm, rarely observed. Pileipellis a cutis, hyphae 3.7–6.3 diam., cylindrical with outgrowths or a few diverticula, thin-walled, weakly encrusted with brown pigments, terminal cells non-encrusted, lobed, pale brown in KOH, inamyloid. Stipe hyphae 5.9–6.4 µm, cylindrical, parallel, thin-walled, hyaline to pale brown in KOH, inamyloid. Caulocystidia 5–6 × 31–54 µm, cylindrical to sinuous, hyaline, thin-walled. Clamp connections present in all tissues.

Material examined: **PAKISTAN**, **Khyber Pakhtoonkhaw**, Mansehra, Dadar, under *Pinus roxburghii*, 15 September, 2012, Malka Saba & Abdul Nasir Khalid, MSM #003 (LAH; GenBank KF803762).

Comments: *Gymnopus menehune* is diagnosed by its convex to plano-convex, umbilicate pileus, pubescent to tomentose stipe, and elongate to ellipsoid basidiospores. According to Desjardin et al. (1999) there are voluminous lobed cheilocystidia in *G. menehune*, but cheilocystidia were rarely observed in our Pakistan collection. The species was described first from Hawaii (Desjardin et al. 1999) and later from Indonesia (Wilson et al. 2004). *Gymnopus menehune* represents a new record for Pakistan.

Gymnopus menehune falls in *G.* subsect. *Vestipedes* (Antonín & Noordeloos 1997).

Phylogenetic analysis

Sequencing of the nrITS region of *Gymnopus luxurians* yielded fragments of 800 base pairs while *G. menehune* yielded 830 base pairs. Initial BLAST analysis of nucleotide sequences showed collections MSM#001 and MSM#002 with 100% and 99% maximum identity with *Gymnopus luxurians* (GenBank AY256709, DQ480106, DQ450022), while collection MSM#003 showed 97% maximum identity with *G. menehune* (GenBank JN182864, AY263426, AY263444, DQ450043, AY263443).

Sequences of closely related taxa were retrieved from Genbank for phylogenetic analysis. In addition to the three new Pakistani sequences of *G. luxurians* and *G. menehune*, the analysis included 30 other *Gymnopus* sequences from GenBank, and three *Omphalotus* sequences as outgroup (Table 1).

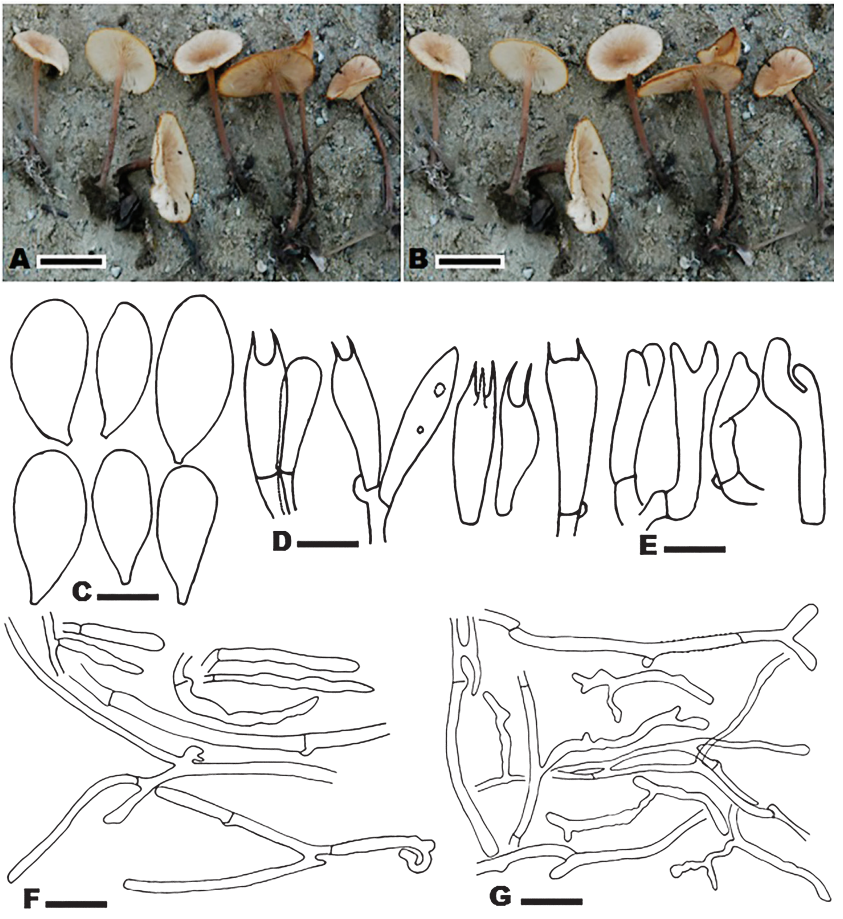

Fig. 2. *Gymnopus menehune* (MSM #003). (A–B) Basidiomes. (C) Basidiospores. (D) Basidia and basidioles. (E) Cheilocystidia. (F) Stipe elements and caulocystidia. (G) Pileipellis. Scale bars: A–B = 2 cm; C–E = 10 μm; F–G = 20 μm.

After removing and editing ambiguities from the aligned sequences, a total of 948 positions were in the final dataset. Of these, 508 characters were conserved, 418 were variable, 380 were parsimony informative, and 32 were singletons.

Clade I, and II (Fig. 3) included species of *Gymnopus* subsect. *Vestipedes*, Clade III species characteristic of *G.* sect. *Gymnopus*, and clade IV species of *G.* subsect. *Levipedes*. Pakistani sequences (▪) generated in this study clustered with their respective morphological species, each one with strong bootstrap value. *Gymnopus luxurians* appears in a clade distinct from *G. menehune*.

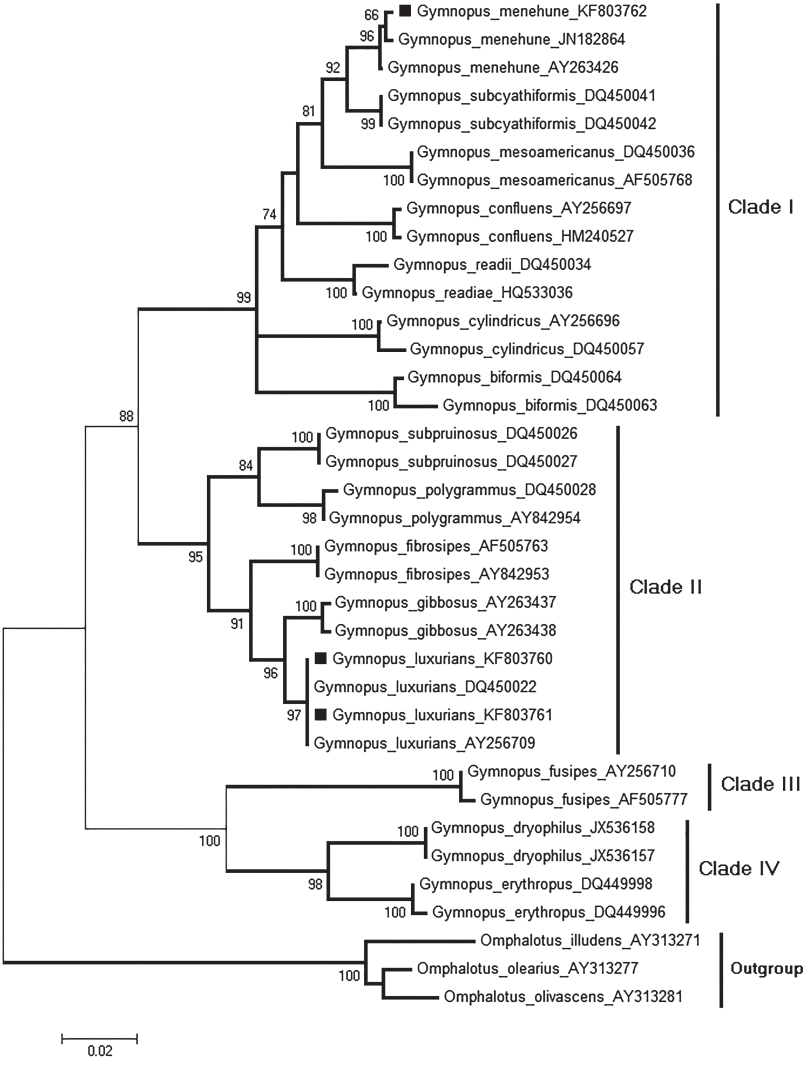

Fig. 3. Molecular phylogenetic analysis of *Gymnopus* sequences by the Maximum Likelihood method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history. The percentage of trees in which the associated taxa clustered together is shown next to the branches. New sequences reported in this study (▪).

The percentage similarity, calculated by DNAStar, of *Gymnopus luxurians* showed 100% identity and 0% genetic divergence with *G. luxurians* sequences AY256709 and DQ450022, and *G. menehune* showed 99.9% identity and

0.1% divergence with *G. menehune* sequences JN182864 and AY263426; this supports the results of the BLAST analysis and the phylogenetic tree.

Key to the *Gymnopus* spp. in Pakistan

1. Pileipellis a transition between cutis and trichoderm and comprising lobed (often coralloid) elements, similar to a Dryophila-structure ..... *G. fusipes*
1. Pileipellis usually a simple cutis with some weakly to distinctly coralloid or diverticulate terminal elements, without Dryophila-structure 2
2. Stipe smooth 3
2. Stipe hairy or tomentose 4
3. Pileus dark red brown at centre, much paler yellow brown to yellow red towards margin ..... *G. erythropus*
3. Pileus with ochre brown tinges, especially at centre, translucently striate up to half the radius ..... *G. dryophilus*

4a. Pileus convex when young, expanding with age to plane, flat, thin (collybioid), slightly umbonate; lamellae adnexed, close to subdistant; basidiospores oblong to ellipsoid, 4–5 × 5.5–9.5 µm ..... *G. luxurians*

4b. Pileus convex to plano-convex and slightly umbilicate when young, infundibuliform, umbilicate at maturity; lamellae adnate to decurrent, close; basidiospore elongate to ellipsoid, 3–5.5 × 5.5–9 µm ..... *G. menehune*

4c. Pileus convex with an incurved margin when young, becoming broadly convex, bell shaped or nearly flat; lamellae narrowly attached to the stem or free, crowded or close; basidiospores lacrymoid to elliptical or nearly fusoid, 3.5–5 × 6–10 µm ..... *G. confluens*

4d. Pileus convex then flattened, often with a low, broad umbo, tan to darker brown; lamellae adnexed to free, crowded; basidiospores elongate, 3–4 × 7–9 µm ..... *G. peronatus*

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